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**13. Abstract** *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Sphingosine kinase 1 (SphK1) and its product sphingosine 1-phosphate (S1P) have been shown to promote cell growth and inhibit apoptosis of tumor cells (reviewed in (1)). Moreover, sphingosine kinase has been shown to be responsible for radioresistance of certain prostate cancer cells (2). In an effort to understand the regulation of SphK1, we undertook a two-hybrid screen for SphK1-interacting proteins. We found eleven potential interactors. Over the course of the report period, we have focused our efforts on one of these interactors, aminoacylase 1 (Acy1). Acy1 is a metalloprotein that removes amide-linked acyl groups from amino acids, and may play a role in regulating oxidative damage. We have shown that both the C-terminal fragment and full length Acy1 co-immunoprecipitate with SphK1. Both proteins also cause a redistribution of SphK1 as observed by immunocytochemistry. Though both C-terminal and full length proteins reduce SphK1 activity measured *in vitro*, the C-terminal fragment inhibits while the full length potentiates the effects of SphK1 on proliferation and apoptosis. Thus, full length Acy1 physically and physiologically interacts with SphK1. Moreover, the dominant-negative activity of the C-terminal fragment of Acy1 may suggest potential clinical targets for specific inhibition of SphK1 activity.

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# Introduction

Sphingolipids are ubiquitous constituents of eukaryotic membranes characterized by the presence of an acylated sphingoid base, ceramide (Cer). Cer and its further metabolites sphingosine (Sph) and Sph-1-phosphate (S1P) are now recognized as potent bioactive molecules. In many cell types, increased Cer and Sph levels lead to cell growth arrest and apoptosis (reviewed in (1, 3, 4)). Conversely, S1P promotes cell growth and inhibits apoptosis (reviewed in (1, 5, 6)). Cells contain signal-regulated enzymes that can interconvert Cer, Sph, and S1P. Thus, conversion of Cer and Sph to S1P simultaneously removes pro-apoptotic signals and creates a survival signal, and vice versa. This led to the proposal of a "sphingolipid rheostat" as a factor determining cell fate (7). According to this hypothesis, it is not the absolute levels but the relative amounts of these antagonistic metabolites that determines cell fate. In agreement, it has been shown that increased S1P protects against Cer-induced apoptosis, and depletion of S1P enhances Cer-induced apoptosis (7-10).

There are a number of agonists, especially growth and survival factors, that have been reported to increase SphK activity, including ligands for G-protein coupled receptors (GPCRs) (11-13) and growth factor receptors (8, 14, 15). Activation of SphK is required for at least some of the signaling effects observed. Requirement for SphK activation was typically based on the ability of inhibitors of SphK, including dominant negative SphK1 (16), to block agonist-induced effects and/or the ability of exogenously added S1P or a precursor to bypass the agonist. While many early studies suggested a role for S1P as an intracellular second messenger, it was later demonstrated that S1P is also a ligand for a family of GPCRs (reviewed in (17)). Complicating matters, there is growing evidence that agonist-induced SphK activation leads to S1P secretion (18, 19) and autocrine and/or paracrine signaling to the cell surface S1P receptors (20, 21).

SphK1 and S1P have been linked to growth, metastasis, and radio- and chemotherapy resistance of tumors, including prostate tumors (reviewed in (1)). For example, it was shown that in radiation sensitive prostate cancer cells, γ-irradiation reduces SphK1 activity, leading to increased Cer and Sph levels and subsequent apoptosis. However, radiation-resistant prostate cancer cells showed no change in SphK activity or Cer levels. Furthermore, inhibitors of SphK sensitized these cells to γ-irradiation, demonstrating a role for SphK in prostate tumor radiation resistance (2).

In order to better understand the regulation and activation of SphK1, we had performed a two-hybrid screen for protein interactors of SphK1. In the initial proposal, we set out to characterize several of these interactors and their potential physiological influence on SphK1. Here we report our results investigating one of these interactors, aminoacylase 1 (Acy1).

# Updated Results

SAGE analysis (http://www.ncbi.nlm.nih.gov/SAGE/) reveals that the expression of Acy1 is down regulated upon androgen treatment of prostate cancer cells. This protein has been characterized as a cytosolic enzyme of amino acid salvage (22), catalyzing the hydrolysis of amide-linked acyl chains of amino acids. Because the two-hybrid screen yielded only C-terminal third of Acy1, we cloned by PCR the full length protein from a mouse kidney cDNA library and inserted a V5 epitope tag (**Task 1d**; data not shown). We then expressed both proteins in HEK 293 cells and determined that full length Acy1, like the C-terminal fragment from the two-hybrid screen, co-immunoprecipitated with SphK1 (**Task 1c,d**; figure 1). We then expressed this construct in HEK 293 cells to see if it altered the properties of SphK1. SphK assays were performed on extracts of HEK 293 cells expressing either vector or SphK1 and either vector, the C-terminal fragment of Acy1 or full length Acy1 (**Task 1e** figure 2a). Interestingly, expression of either construct slightly decreased SphK1 activity measured *in vitro*. We then checked the localization of the SphK1 and Acy1 by western analysis (figure 2b). The extracts were separated into three fractions: cytosolic (Cy), Triton X-100 soluble membranes (TS), and Triton X-100 resistant membranes (TI). Interestingly, when co-expressed with Acy1, a portion of SphK1 shifted from the cytosol to the Triton X-100 soluble membranes.

These results also suggested that SphK1 and Acy1 physically interact *in vivo*. Thus we looked at the localization of both proteins by immunocytochemistry. Acy1, SphK1, or both were expressed in Cos 7 cells and stained with the corresponding epitope tags (**Task 1c,d**; figure 1). When expressed alone, Acy1 had a diffuse cytosolic staining as predicted from the literature and fractionation data (figure 2b). When SphK1 was expressed alone, it also showed a diffuse cytosolic expression pattern with dispersed punctate staining as reported previously (23). However, when the two proteins were expressed together, we observed not only co-

localization of the two proteins but also the appearance of tubular structures, likely membraneous (figure 3, Appendix).

**Figure 1: SphK1 co-immunoprecipitates Acy1.** HEK cells were transfected with V5-Acy1 and either vector or myc-SphK1 using Lipofectamine Plus (Invitrogen) as per manufactures instructions. Lysates were prepared and immunoprecipitated with anti-myc (SphK1) antibodies. The immunoprecipitates were washed and resolved by SDS-PAGE. The gel was blotted to nitrocellulose and probed with anti-V5 (Acy1). Only when myc-SphK1 is present is Acy1 detected in the immunoprecipitation lane.
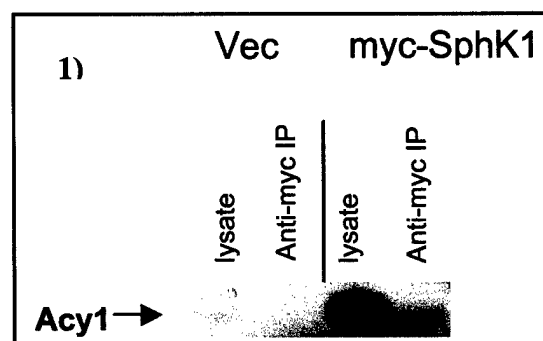


**Figure 2: ACY1 alters the subcellular localization of SphK1.** (A). HEK-293 cells were transfected with vector or SphK1 and either vector, the C-terminal fragment of Acy1, or full length. Lysates were then prepared and assayed as described for SphK1 activity (23). (B). HEK-293 cells were transfected with vector, myc-SphK1, and/or V5-ACY1. Cells were then fractionated as into cytosolic (Cy), 1% Triton X-100 soluble membranes (TS), and 1% Triton X-100 insoluble membranes (TI). Westerns were then performed to determine the relative distribution of SphK1 in these fractions.
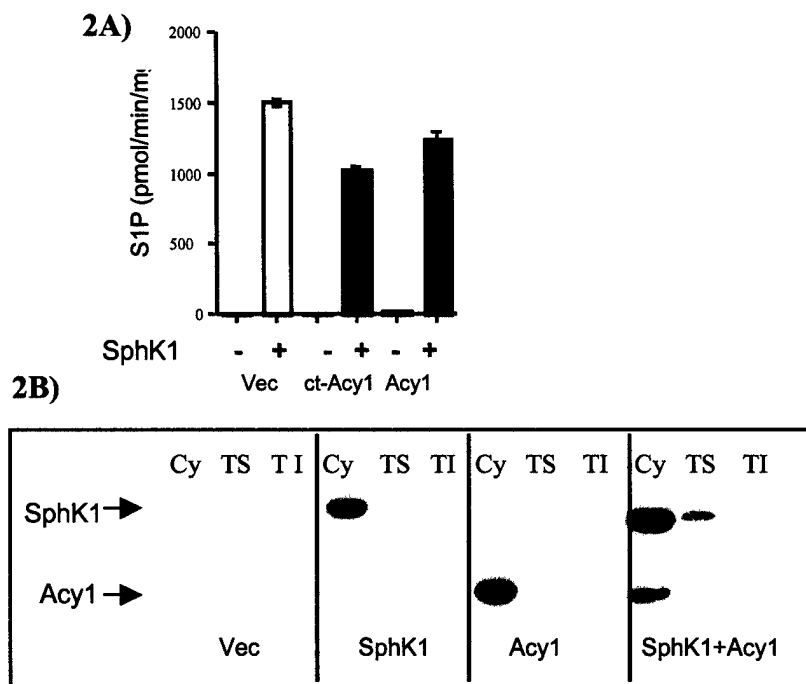


Acy1 has a neutral pH optimum and has been shown to catalyze the reverse reaction, the addition of amide-linked acyl chains in a CoA-independent manner (24). All of these properties are similar to the purified cytosolic ceramidase activities (25). Because the product of ceramidase, Sph, is a substrate for SphK1, we hypothesized that Acy1 might be a ceramidase. Interaction of a ceramidase and an SphK would be a convenient way to coordinately regulate the enzymes. To test this hypothesis **(Task 2d)**, I performed the standard SphK1 assay in cell extracts overexpressing both SphK1 and Acy1 under conditions reported to be favorable for the activity of both enzymes. However, instead of using Sph as a substrate, I used Cer, reasoning that if Acy1 were a ceramidase, it would generate Sph that SphK1 would phosphorylate. However, the assays showed no increase in S1P in extracts expressing both SphK1 and Acy1 versus SphK1 alone (data not shown).

Still, the above results demonstrate that Acy1 and SphK1 physically interact. Therefore we next tested whether Acy1 physiologically interacted with SphK1. It is known that SphK1 over-expression promotes cell growth and transition through the cell cycle (23). We performed MTT assays of cell growth in NIH 3T3 fibroblasts expressing either vector or SphK1 and either vector, the C-terminal fragment of Acy1 or full length Acy1 **(Task 2c)**. We included the fragment because of the possibility that it may act as a dominant negative inhibitor of Sphk1: the C-terminal fragment of Acy1 binds SphK1 but lacks residues critical for Zn binding, which is known to be necessary for Acy1 activity (26). And in fact, this is just what we observed. SphK1 alone increased the growth of cells relative to vector (figure 4), while the C-terminal fragment blocked this effect. Moreover, expression of full length Acy1 enhanced the SphK1 effect on the growth rate of cells. These data suggest that Acy1 and SphK1 work together to promote cell growth, and that the C-terminal fragment of Acy1, acts as a dominant negative regulator of SphK1 activity on cell growth.

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**Figure 4: Acyl potentiates while the C-terminal fragment attenuates SphK1-induced cell growth.** NIH 3T3 fibroblasts were plated at equal numbers and transfected with the indicated plasmids. 48 h later the cells were assayed for reduction of MTT, a measure of cell growth, using the MTT assay kit from Calbiochem as per manufactures instructions
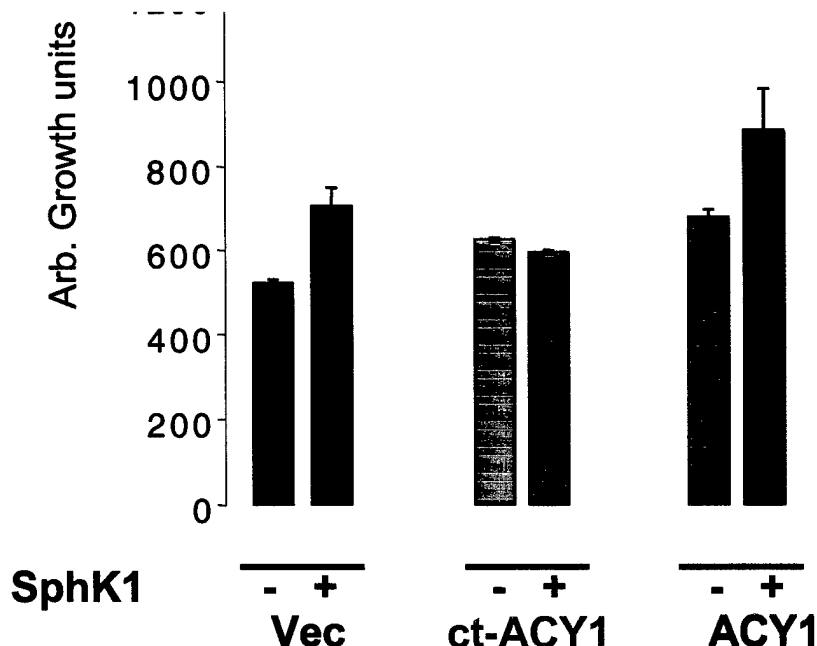
Another well characterized effect of SphK1 is the protection it provides against apoptosis (7). We therefore investigated possible role of Acyl in SphK1-induced protection from apoptosis in a serum-deprivation model in NIH 3T3 cells (23). Cells were again transfected with either vector or SphK1 and either vector, the C-terminal fragment of Acyl or full length Acyl (**Task 2c**). Cells were then deprived of serum for 24 h and the amount of apoptosis assessed by observation of nuclear condensation and fragmentation (figure 5). As expected, SphK1 protected cells from apoptosis. Full length Acyl slightly potentiated the SphK1-protection. Strikingly, the C-terminal fragment of Acyl had little effect on apoptosis alone but almost completely blocked the ability of SphK1 to protect against apoptosis. These results further substantiate a role for Acyl in the regulating the physiological activities SphK1 and again demonstrate that the C-terminal fragment of Acyl acts as a dominant regulator of SphK1.



**Figure 5: Acyl potentiates while the C-terminal fragment attenuates SphK1 protection from apoptosis.** NIH 3T3 fibroblasts were plated at equal numbers and transfected with the indicated plasmids. 24 h later the cells were starved for serum for an additional 24 h. After that time, they were fixed on the dish and the nuclear condensation and fragmentation was assessed using Hoechst dye. At least 300 cells were counted for each transfectant.
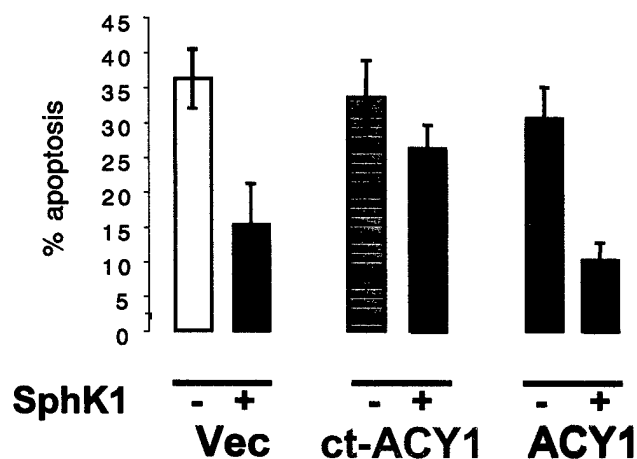
## Key Research Results

- SphK1 physically interacts with both the C-terminus of Acyl as well as full length.
- Acyl alters the intracellular distribution of SphK1.
- Acyl potentiates SphK1 stimulation of cell growth and inhibition of apoptosis.
- The C-terminus of Acyl acts as a dominant negative inhibitor of SphK1-induced cell growth and inhibition of apoptosis.

## Reportable Outcomes

- Speaker, 37th Annual Southeastern Regional Lipid Conference. Cashier, NC, Nov. 6-8, 2003.

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"Sphingosine Kinase 1 interacting proteins modulate its growth-promoting and anti-apoptotic effects."
- Presenter, 2002 Daniel T. Watts Research Symposium. Richmond, VA, Oct.14, 2002. "Aminoacylase 1 Interacts with Sphingosine Kinase 1 to Modulate Its Growth-Promoting and Anti-Apoptotic Effects."
- Invited Speaker, Lipid Signalling: Cellular Events and Their Biophysical Mechanisms. Madrid, Spain, May 20-22, 2003. "Sphingosine-1-Phosphate, an Important Lipid Mediator."

## Conclusion

The data accumulated over the period covered in the first reporting period strongly suggests that SphK1 physically interacts with the C-terminal third of Acy1. Moreover, the Acy1 physiologically interacts with SphK1, regulating both its pro-growth and anti-apoptotic effects. While it is still formally possible that SphK1 and Acy1 act in parallel growth-promoting and apoptosis-inhibiting pathways, the weight of the data showing that the proteins physically interact and that Acy1 effects two independent effects of SphK1 strongly suggests that Acy1 is a bona fide physiological regulator of SphK1. These results also suggest that the C-terminus of Acy1 acts as a dominant negative inhibitor of SphK1-induced cell growth and inhibition of apoptosis. Thus, further characterization of the SphK1-Acy1 interface may provide a target for inhibition of the tumor promoting activities of SphK1.

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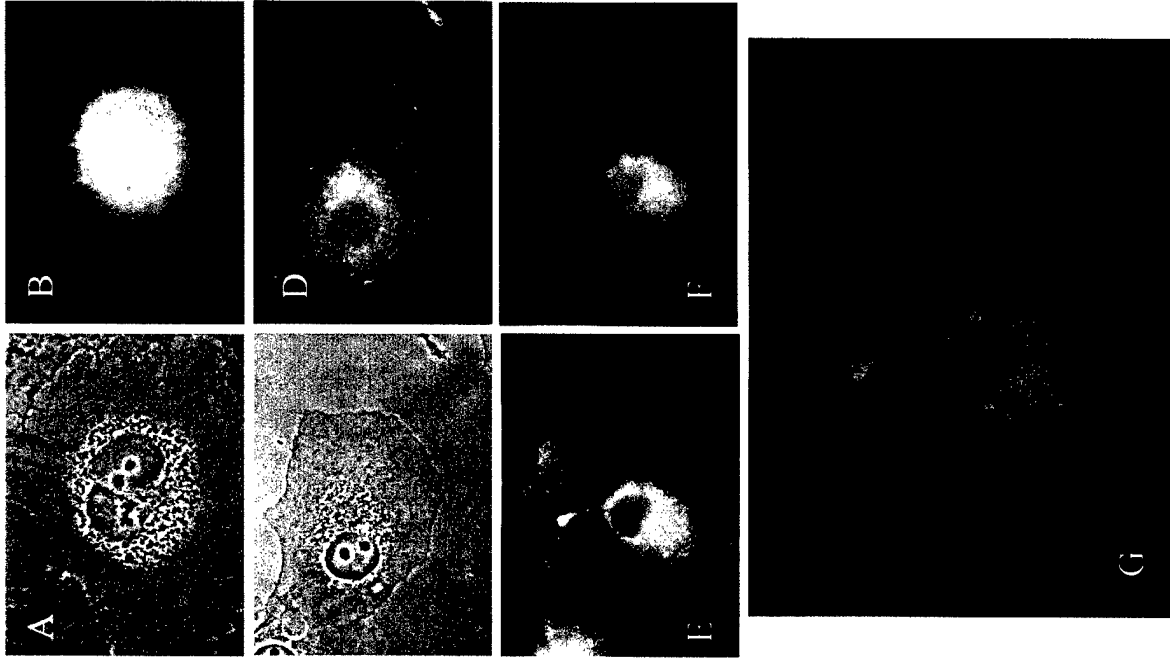
**Figure 3: Acy1 alters the distribution of SphK1.** Cos7 cells were tranfected via Lipofectamine 2000 (Invitrogen) per manufactures instructions with either Acy1 (A,B), SphK1 (C,D) or both (E-G). 24h later the cells were fixed and stained with the appropriate antibodies. Phase (A,C) and epifluorescent (B,D-G) images were taken at 60x. Image G is a merge of E (SphK1, red) and F (Acy1, green).